

ORIGINAL ARTICLE

Development and comparison of SYBR Green quantitative real-time PCR assays for detection and enumeration of sulfate-reducing bacteria in stored swine manure

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Abstract

Aims: To develop and evaluate primer sets targeted to the dissimilatory sulfite reductase gene (*dsrA*) for use in quantitative real-time PCR detection of sulfate-reducing bacteria (SRB) in stored swine manure.

Methods and Results: Degenerate primer sets were developed to detect SRB in stored swine manure. These were compared with a previously reported primer set, DSR1F+ and DSR-R, for their coverage and ability to detect SRB communities in stored swine manure. Sequenced clones were most similar to *Desulfovibrio* sp. and *Desulfohalobus* sp., and these SRB populations differed within different manure ecosystems. Sulfur content of swine diets was shown to affect the population of *Desulfohalobus*-like Group 1 SRB in manure.

Conclusions: The newly developed assays were able to enumerate and discern different groups of SRB, and suggest a richly diverse and as yet undescribed population of SRB in swine manure.

Significance and Impact of the Study: The PCR assays described here provide improved and efficient molecular tools for quantitative detection of SRB populations. This is the first study to show population shifts of SRB in swine manure, which are a result of either the effects of swine diets or the maturity of the manure ecosystem.

Introduction

The intensification of large-scale livestock production has led to the consolidation of large amounts of animal manure into fewer and more confined locations. A consequence of concentrated manure storage, such as the deep pit storage method, is the production of a variety of malodorous chemicals. Odour emanating from swine production facilities has led to increased tension among rural neighbours and can influence zoning decisions about the proposed sites of new pig production operations. Nuisance odour from stored swine manure results largely from compounds such as sulfides, volatile fatty acids, phenols, indols (e.g. skatole) and carbonyls, which are products of incomplete anaerobic digestion of protein and carbohydrate (Zahn *et al.* 1997; Sutton *et al.* 1999). These compounds are not only responsible for unpleasant odours but can also pose other problems, affecting the

comfort, health and production efficiency of the animals, as well as the health and comfort of human workers. Hydrogen sulfide (H₂S) and other sulfur compounds comprise about one-half of the offensive odorants from swine manure (Clark *et al.* 2005a). H₂S is not only extremely odorous, but when the slurry is disturbed during removal by agitation and pumping, the sudden release of H₂S can raise concentrations to dangerously toxic levels (Donham 2000).

Production of sulfur compounds by anaerobic bacteria involves sulfate reduction and metabolism of sulfur-containing amino acids. In dissimilatory sulfate reduction, sulfate is used as a terminal electron acceptor and hydrogen sulfide is produced. A key enzyme in this pathway is dissimilatory sulfite reductase (DSR), which catalyses the reduction of (bi)sulfite to sulfide, the final, central energy-conserving step of sulfate respiration (Odom and Peck 1984). The DSR enzyme has been shown to be

evolutionarily conserved among sulfate-reducing bacteria (SRB) (Wagner *et al.* 1998). The gene encoding the α -subunit of this key metabolic enzyme (*dsrA*) has become a promising target, as an alternative to 16S rRNA genes, for molecular based approaches to detect and enumerate SRB in different environments. Quantitative PCR-based detection methods targeting amplification of *dsrA* as a means for enumerating SRB have been developed and applied for use in different natural environments such as marine sediments (Perez-Jimenez *et al.* 2001; Kondo *et al.* 2004; Kjeldsen *et al.* 2007; Leloup *et al.* 2007), soda lakes (Foti *et al.* 2007), microbial mats (Minz *et al.* 1999) and hydrothermal vents (Dhillon *et al.* 2003). These molecular methods have not yet been readily applied to investigate and identify SRB in stored manure, despite the importance of H₂S production in this environment. Only recently has an initial study by Cook *et al.* (2008), addressed the need for identification and enumeration of SRB in stored swine manure by using a phylogenetic and molecular-based approach.

Research on microbial population ecology in livestock has focused mostly on organisms in the rumen or swine gastrointestinal tract (Whitford *et al.* 1998; Leser *et al.* 2002; Hill *et al.* 2005; Tajima *et al.* 2007). A few studies have begun to use molecular analysis to identify isolates and characterize 16S rRNA gene clones from swine manure (Cotta *et al.* 2003; Whitehead and Cotta 2004; Whitehead *et al.* 2004, 2005). However, there are very few studies that have used molecular techniques to identify specific microbial populations responsible for odour production in swine manure. A recent study by Cook *et al.* (2008), began to uncover the myriad diversity of SRB in stored swine manure using a phylogenetic and molecular based approach targeting the *dsrA* gene. This was the first such study designed to target specific microbial groups responsible for odour production, and more specifically, to evaluate the phylogeny and concentration of SRB in swine manure using molecular methods. Phylogenetic analysis of cloned *dsr* genes identified three major groups of SRB in swine manure with similarity to *Desulfobulbus* and *Desulfovibrio*-like species. A TaqMan[®] probe-based approach was used to target and enumerate these organisms using quantitative real-time PCR. Further studies in our laboratory suggested that this assay was only detecting a small proportion of the SRB in any one group. Of the 82 clones sequenced in the Cook study, only 54% of these SRB could be detected using the TaqMan[®] probe-based quantitative PCR assays. This illustrates the need to design new degenerate primer sets to target the *dsrA* gene and encompass all the clones of each particular group.

A *dsrA*-amplifying primer set (DSR1F+ and DSR-R) developed by Kondo *et al.*, has been used recently to detect and enumerate SRB populations in marine sedi-

ment by quantitative real-time PCR (Kondo *et al.* 2004; Leloup *et al.* 2007). One-hundred and twelve out of 356 *dsrAB* sequences, have perfectly matched target sites for the primers in this assay, mostly from *Desulfovibrionaceae* and *Desulfobacteraceae* (Leloup *et al.* 2007). This primer set has not previously been utilized to identify and enumerate SRB populations in stored swine manure.

As much of the malodour from stored swine manure results from incomplete anaerobic digestion of protein and carbohydrate, one method widely used to reduce the quantity of volatile emissions from swine facilities is swine-diet manipulation (Sutton *et al.* 1999; Clark *et al.* 2005b; Panetta *et al.* 2006). Little is known, however, about the impact these changes in diet have on the micro-organisms present in the stored manure. Dietary changes that lead to differences in the microbial population in the stored manure may have direct consequences on the generation of odour. In order to determine the effect of diet on SRB population in stored swine manure, and in particular the effect of altering sulfur levels in the diet, SRB were quantified in manure generated from pigs consuming diets with altered levels of sulfur.

The objectives of this study were to develop more reliable quantitative real-time PCR techniques, using degenerate primers targeting 100% of previously cloned *dsrA* genes, to quantify and evaluate the diversity of the SRB population in stored swine manure. A primer set previously used to identify SRB in marine environments was adapted and modified for enumeration of SRB in stored swine manure, and clone libraries were constructed to determine the diversity of coverage. These new assays were applied to detect differences in the SRB population in response to diet manipulation.

Materials and methods

Swine manure samples and swine diets

Swine manure was collected from underground storage pits at two separate swine farms (Farm 1 and 2) in Illinois. Pigs were fed a corn-soybean based diet. Three manure samples were collected from each pit at c. 1 m depths using a Tank Sampler (NASCO, Fort Atkinson, WI, USA), transferred to Whirl-Pak sampling bags (NASCO) and kept on ice while returning to the laboratory. Individual samples were mixed to form a composite manure sample representative of each farm. Diet studies were performed by Brian Kerr at the National Soil Tilth Laboratory, Swine Odor and Manure Management Research Unit, ARS-USDA, Ames, Iowa, as part of a larger research investigation analysing effects of diet on swine performance. Pigs were fed a regular corn-soybean based diet, with a sulfur content of 2293 mg kg⁻¹

(Diet 1) or a low sulfur content of 1918 mg kg⁻¹ (Diet 2). There were three pigs per diet, and manure (faecal matter, urine, spilled food and water) was collected continuously for the duration of the dietary feeding period (28 days). At the end of the study, a 50 ml sample of manure from each dietary feeding was collected and frozen at -20°C. Manure samples were shipped frozen to our laboratory for analysis of the SRB population.

DNA extraction from swine manure

A 1 ml sample of swine manure was mixed with an equal volume of Tris-saturated phenol and 10% v/v zirconium beads (0.1 mm). Samples were homogenized for 30 s at a speed of 4 m s⁻¹ in a FastPrep® Instrument (Q-BIOgene, Irvine, CA, USA), followed by centrifugation for 10 min at 14 000 g. A 600 µl aliquot of the supernatant was then added to 500 µl Fastprep® Binding Matrix and extracted using the FastDNA® Spin kit (Q-BIOgene) according to the manufacturer's instructions.

Real-time PCR analysis

Real-time PCR assays were performed on a Rotorgene 6000 (Corbett Robotics Inc., San Francisco, CA, USA), using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA). Template DNA (5 µl) was used in a reaction mixture containing 10 µl 2 × QuantiTect SYBR Green PCR Mastermix, 500 nmol l⁻¹ of each forward and reverse *dsrA* primer (Table 1), and H₂O to a final volume of 20 µl. Reaction conditions were 95°C for 15 min (1×), then 95°C, 15 s, 59°C, 30 s, 72°C, 30 s (40×). A final melt curve analysis was performed to determine the presence or absence of nonspecific amplification products. For quantification of 16S rDNA, 5 µl template DNA was used in a reaction mixture containing 10 µl 2 × QuantiTect Probe PCR Mastermix (Qiagen), 500 nmol l⁻¹ of each forward (1055F) and reverse (1392R) primer,

250 nmol l⁻¹ probe (16STaq1115-BHQ) and H₂O to a final volume of 20 µl. Reaction conditions for quantification of 16S rDNA were 95°C for 15 min (1×), then 95°C, 15 s, 58°C, 60 s (40×). All samples were run in triplicate and H₂O replaced template in control reactions.

Standard curves

For each of the three group *dsrA* assays, standard DNA consisted of plasmid carrying a *dsrAB* insert from previously sequenced cloned slurry samples (Cook *et al.* 2008). Standard DNA for determination of total SRB using the Kondo *dsrA* primer set was a cloned *dsrAB* gene from a Group 1, *Desulfobulbus*-like clone: L-5S-5, which has 81% identity to *Desulfobulbus elongatus* (AF418202) at the amino acid level (Cook *et al.* 2008). The forward and reverse Kondo *dsrA* primers had an exact match to this template and the amplified product was of the expected size (221 bp). Bacterial 16S rRNA gene copy numbers were determined as previously described (Harms *et al.* 2003). Standard DNA consisted of a plasmid carrying a 16S rRNA gene sequence from a swine manure isolate (AY167932) (Whitehead and Cotta 2001). Standard curves were generated from different concentrations of template DNA using the respective real-time PCR programme for each primer set. All standards were run in triplicate. Amplification efficiencies and melting curves were determined and analysed using Rotor-Gene 6000 series software (Corbett Robotics Inc.).

Comparative *dsrA* sequence analyses

A clone library was constructed using *dsrA*-PCR products and the TOPO® TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, DNA was extracted from swine manure and the *dsrA* gene was PCR amplified with primers DSR1F+ and DSR-R as described for real-time PCR analysis. The

Table 1 Oligonucleotide primers and probes used in this study for quantitative real-time PCR

Primer	Sequence (5'-3')	Length (bp)	Amplicon length (bp)	Reference
Grp1fd	GYGAGTGGKCCCTGCTAYGA	19	172	This study
Grp1rd	CCAGGTGCCGATAACRGC	18		
Grp2fd	CGACACCCARGACATGTGC	19	121	
Grp2rd	GCWGTACGCAACCGTTGGG	20		This study
Grp3fw	CTGCGAATATGCCTGTACA	20	119	
Grp3rd	GGGGCARCCGTCGAACTTG	19		
Dsr1F+	ACSCACTGGAAGCACGGCGG	20	221	Kondo <i>et al.</i> (2004)
Dsr-R	GTGGMRCCTGTCAKRTTGG	19		
1055F	ATGGCTGTCGTCAGCT	16	337	Harms <i>et al.</i> (2003)
1392R	ACGGGCGGTGTGTAC	15		
16sTaq1115-BHQ	(6-FAM)-CAACGAGCGCAACCC-(TAMRA)			Cook <i>et al.</i> (2008)
SRB-DSR-BHQ	(6-FAM)-CCGGCYTCCCTACAAG-(TAMRA)			

221 bp amplified product from Farm 1 and Farm 2 manure were ligated into the TOPO® TA cloning vector. Randomly selected clones were screened for inserts of the expected size using universal M13 forward (5'-GTAAACGACGGCCAG-3') and reverse (5'-CAG-GAAACAGCTATGAC-3') primers, and *dsrA* inserts were sequenced at the W. M. Keck Center for Comparative and Functional Genomics (University of Illinois Biotechnology Center, Urbana, IL, USA) with an M13 forward sequencing primer. A total of 20 Farm 1 manure clones and 20 Farm 2 manure clones were sequenced. DNA sequence homology analysis was performed using the National Center for Biotechnology Information BLAST database searches. Sequence alignment and phylogenetic analysis were performed using MEGA4 (Tamura *et al.* 2007). The neighbour-joining method was used to infer phylogenetic relationships between clones and known organisms.

Results

Design of real-time PCR primers

In a previous study designed to target specific microbial groups responsible for odour production, Cook *et al.* (2008) cloned and sequenced 82 *dsr* genes isolated from stored swine manure. Based on sequence alignments and amplification results, only 54% of sequenced clones were detected using the primers and probes designed for use in quantitative real-time PCR in that study. Further studies in our laboratory identified the need to design improved primer sets to encompass all the clones in each group, thereby increasing the accuracy and reliability of quantifying SRB from swine manure. A multiple alignment of previously cloned *dsrA* genes was constructed using a CLUSTALW program (Tamura *et al.* 2007). Based upon these alignments, appropriate sites for *dsr*-targeted oligonucleotide primers were chosen. Degeneracies were introduced into the primer design to allow for amplification of all clones in each respective group. The new designated primers for each group are shown in Table 1. These are all improved primer sets of the previously published QDSR403B-F and QDSR532B-R (Group 1A), QDSR-G1Bf and QDSR532Br (Group 1B), QDSR390V-F and QDSR503V-R (Group 2A), and QSEC425-F and QSEC527-R (Group 3A) (Cook *et al.* 2008). These new primer sets target, and have fewer mismatches to, a larger proportion of clones. Primer set target regions of group 1, 2 and 3 *dsrA* genes are shown in Fig. 1. Amplification of all clones within Groups 1A, B and C is now attainable with the new Grp1fd and Grp1rd primer sets. This primer set also specifically targets the *dsrA* gene of *Desulfobulbus propionicus* (AF218452) and *Desulfobulbus elongatus* (AF418202), the two closest known SRB to the

Group 1 isolates based on sequence homology analysis of *dsrA* (Fig. 1). The Group 2A and B clones did not group with any known SRB, but the target regions of Grp2fd and Grp2rd correspond to all clones within both Groups 2A and B (Fig. 1). The closest similarity of known SRB, based on *dsrA* homology, to the Group 3A and B clones were *Desulfovibrio desulfuricans* (AF273034) and *Desulfovibrio intestinalis* (AF418183). The *dsrA* gene of these strains, as well as all clones within Groups 3A and B, are now targeted by primer set Grp3fw and Grp3rd (Fig. 1).

A SYBR Green-based quantitative real-time PCR assay was developed to eliminate the use of a TaqMan® probe and circumvent the problem of probe mismatches among clones within and between different groups. An advantage of using SYBR Green in quantitative real-time PCR is the ability to perform melt curve analysis, which can detect the presence or absence of nonspecific amplification of target DNA.

A recent study by Leloup *et al.* (2007) utilized a *dsrA*-amplifying primer set to quantify SRB in sulfate and methane zones of the Black Sea marine sediment using quantitative SYBR Green real-time PCR. To date, for the purpose of quantitative real-time PCR, this primer set has the most comprehensive coverage of target *dsrA* genes from known SRB, although they have only been applied to quantify SRB in estuarine/marine environments (Kondo *et al.* 2004; Leloup *et al.* 2006, 2007). We have adapted the Leloup assay and utilized DSR1F+ and DSR-R in a SYBR Green real-time PCR assay to target the *dsrA* gene for enumeration of total SRB in swine manure.

Development and optimization of real-time PCR assays

Standard curves for each primer set were generated by serially diluting cloned target *dsrA* genes representative of each group (Cook *et al.* 2008). Primers specifically amplified a 172, 121 and 119 bp target fragment of *dsrA* respective of Group 1, Group 2 or Group 3. Fragment size was confirmed by agarose gel electrophoresis (data not shown). A standard curve for total SRB enumeration using DSR1F+ and DSR-R was generated using a cloned *dsrA* gene from a Group 1 clone, L-5S-5 (Cook *et al.* 2008). The forward and reverse primers had an exact match to this template and the amplified product was of the expected size (221 bp). The linear range of detection for each of the real-time PCR assays was at least six orders of magnitude from 1×10^2 to 1×10^8 copies per PCR (Fig. 2).

The regression coefficient (r^2) values for standard curves for all real-time PCR assays in each run were always above 0.99 (Fig. 2). Amplification efficiencies (calculated using the equation $E = [10(-1/\text{slope}) - 1] \times 100$

Grp1fd		Grp1rd	
Primer	5' -GYGAGTGG K CCTGCTAYGA-3'	3' -CCAGGTGCCGATAAC R GC-5'	
Target	5' -GYGAGTGG K CCTGCTAYGA-3'	5' -GGTCCACGGCTATTG Y CC-3'	
Group1A	.C.....T.....T..C..	
Group1B	.C.....G.....C..T..	
Group1C	.T.....T.....T..C..	
<i>D. propionicus</i>	.C.....G.....C..C..	
<i>D. elongatus</i>	.C.....G.....C..C..	
Grp2fd		Grp2rd	
Primer	5' -CGACACCCAR R GACATGTGC-3'	3' -GC W GCTACGCAACCGTTGGG-5'	
Target	5' -CGACACCCAR R GACATGTGC-3'	5' -CG W CGATGCGTTGGCAACCC-3'	
Group2AG.....	..T.....	
Group2BA.....	..A.....	
Grp3fw		Grp3rd	
Primer	5' -CTGCGAATATGCCTGCTACA-3'	3' -GGGGC A RCCGTCGAACCTTG-5'	
Target	5' -CTGCGAATATGCCTGCTACA-3'	5' -CCCCGT Y GGCAGCAAGAAC-3'	
Group3AT.....	
Group3BC.....	
<i>D. desulfuricans</i>C.....	
<i>D. intestinalis</i>C.....	

Figure 1 Alignment of target regions of *dsrA* group primers showing consensus sequence. Periods (.) indicate identities with primer sequence. Degeneracies in primer sequence are shown in bold. Noted reference strains with GenBank accession number: *Desulfohalobium propionicus* AF218452, *Desulfohalobium elongatus* AF418202, *Desulfovibrio desulfuricans* AF273034, *Desulfovibrio intestinalis* AF418183.

for Group 1, 2, 3 and total SRB real-time PCR assays were 92%, 104%, 101% and 97%, respectively, and were determined from PCR amplification of plasmid template carrying a *dsrAB* insert from previously sequenced cloned slurry samples (Cook *et al.* 2008 20/id).

The new degenerate primer sets were designed to encompass the area of the previously designed TaqMan[®] probe (SRB-DSR-BHQ – Table 1) within the *dsrA* gene. When all the clones were realigned, the only Group with complete homology match to the probe was Group 1. The newly developed SYBR Green assay was used to quantify Group 1 SRB in manure from Farm 1, and compared with quantification of Group 1 SRB using Grp1fd and Grp1rd primer sets in a real-time PCR assay using the TaqMan[®] probe SRB-DSR-BHQ. In a SYBR Green real-time PCR assay, the mean number of Group 1 SRB was 3.71×10^6 cells ml⁻¹ slurry, compared with 3.82×10^6 cells ml⁻¹ slurry of Group 1 SRB using the TaqMan[®] probe assay (data not shown). These results suggest that the newly developed primer sets for Group 1, in conjunction with a SYBR Green-based real-time PCR assay were efficient in amplifying this specific population of SRB, and that the specificity of amplification was the same as a probe-based assay. Melt curve analysis of the amplified SYBR Green products revealed an absence of nonspecific target PCR products. We were unable to perform this

type of comparison for Groups 2 and 3 as there were too many probe mismatches within all clones of each Group.

Diversity of coverage of DSR1F+ and DSR-R

To check the amplification specificity of DSR1F+ and DSR-R when applied to swine manure, and to determine the diversity of coverage, sequence analysis of PCR products was performed. A clone library was constructed from the manure-amplified 221 bp *dsrA* products. A total of 40 clones (20 from each farm) were sequenced and homology analysis was performed to determine the phylogenetic relationship of the SRB clones to the most closely related known strains of SRB from the database (Genbank).

Sequenced *dsrA* clones formed two main phylogenetic clusters representing either the majority of Farm 1 clones (16) or Farm 2 clones (13) (data not shown). The cluster of 16 Farm 1 clones had between 83–85% sequence identity to the previously isolated swine manure clone 3S-8C (DQ404297) of the *Desulfovibrio*-like Group 2 SRB (Cook *et al.* 2008). The cluster of 13 clones from Farm 2 had sequence similarity to the previously isolated swine manure clone S24 (DQ404314) (between 80–81% identity) of the *Desulfovibrio*-like Group 2 SRB (Cook *et al.* 2008). Three other clones were closely related to *Desulfovibrio simplex* (AB061541) (95% identity). Two clones were

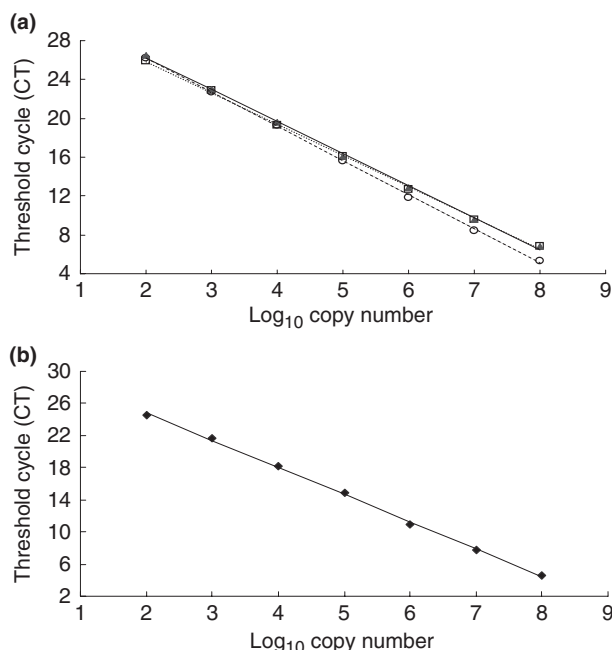


Figure 2 Threshold cycle measurements using Group 1, 2 and 3 (a) and total SRB (b) primer sets on different concentrations of standard template DNA. Data are presented as the mean of triplicate PCR assays. Equation of the line and coefficient of determination are shown for each assay. (○), Group 1: $y = -3.517x + 33.235$ $r^2 = 0.9991$; (□), Group 2: $y = -3.2131x + 32.231$ $r^2 = 0.9985$; (▲), Group 3: $y = -3.294x + 32.838$ $r^2 = 0.9987$; (◆), Total SRBs: $y = -3.3855x + 31.571$ $r^2 = 0.9991$.

closely related to the previously isolated swine manure clone 5S-5 (DQ404302) (88% identity) of the *Desulfobulbus*-like Group 1 (Cook *et al.* 2008). Most of the 40 sequenced clones, including the two main clusters, fall within the *Desulfovibrio* lineage, suggesting a significant diversity of *Desulfovibrio* genera in swine manure that have yet to be isolated and described. Many of the clones are quite distant from known sequences, suggesting that these represent novel and unidentified species.

Quantification of SRB in swine manure

The abundance of SRB in swine manure was investigated using the newly developed real-time PCR assays for quantification of their *dsrA* genes (Fig. 3). Enumeration of total bacteria was performed using quantitative real-time PCR analysis of 16S rRNA genes. It was assumed that the average number of 16S rRNA gene copies per cell was 3.6 (Klappenbach *et al.* 2001), and that the SRB contained only one copy of *dsrA* (Klein *et al.* 2001). Quantification of total bacteria in Farm 1 manure using real-time PCR targeting the 16S rRNA genes estimated a mean total bacterial count of 4.64×10^{10} cells mL^{-1} (data not shown). This is comparable with previous reports of total

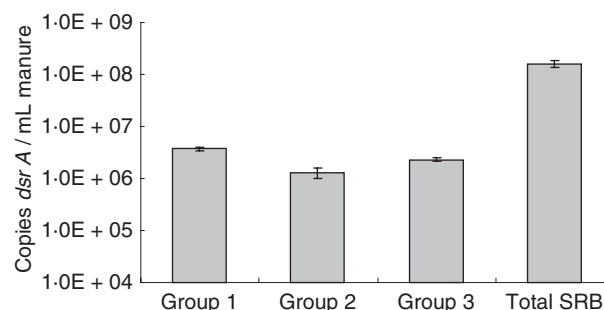


Figure 3 Quantification of SRB in stored swine manure at Farm 1 using SYBR Green quantitative real-time PCR assays. Data are presented as the mean of triplicate PCR assays and error bars represent the standard deviation of the mean.

bacteria, estimated at 1×10^{10} cells mL^{-1} manure (at 1 m depth) by direct microscopic count (Cotta *et al.* 2003). Both methods enumerate both live and dead cells, so data are considered an estimate for total bacterial content, as well as for SRB populations. Figure 3 shows the enumeration of the individual groups of SRB in Farm 1 swine manure, compared with an estimation of the total SRB determined by amplification with DSR1F+ and DSR-R. In Farm 1 slurry, there are 3.71×10^6 cells mL^{-1} Group 1 SRB, 1.28×10^6 cells mL^{-1} Group 2 SRB and 2.31×10^6 cells mL^{-1} Group 3 SRB, with the *Desulfobulbus*-like Group 1 being the slightly dominant population in this sample. There were an estimated 1.6×10^8 cells mL^{-1} total SRB in 1 ml of manure as determined by amplification with DSR1F+ and DSR-R. This is one and a half logs higher than the number of SRB quantified by adding together the total numbers for each Group (a total of 7.3×10^6 cells mL^{-1}), suggesting that the Kondo primer set does actually target a larger SRB population in swine manure.

Effects of diet manipulation on SRB population

The newly developed real-time PCR assays were used to enumerate and identify changes in the SRB population of swine manure in response to altering the sulfur content of the swine diet. As shown in Fig. 4, Group 3 were the predominant SRB, with a total of 5.83×10^6 and 3.46×10^6 cells mL^{-1} for Diets 1 and 2, respectively. The sulfur content of the diets had no detectable effect on the Group 3 SRB population. Likewise, diet sulfur content had no effect on the Group 2 SRB, with a total of 8.9×10^2 and 8.03×10^2 cells mL^{-1} for Diets 1 and 2, respectively. There was, however, an effect on Group 1 SRB in response to lower dietary sulfur content. When dietary sulfur was lower (diet 2), the Group 1 SRB population in the respective manure was 2.79×10^2 cells mL^{-1} , compared with 2.62×10^3 cells mL^{-1} in the control diet 1.

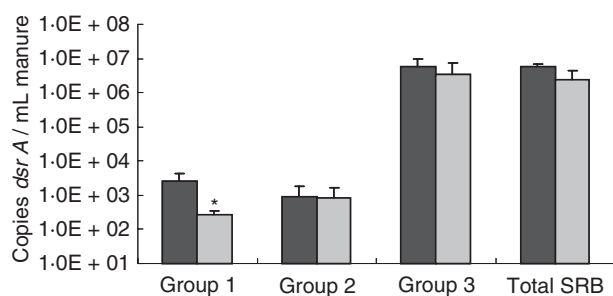


Figure 4 Effects of dietary sulfur concentration on SRB population in stored swine manure. ■ Diet 1: standard sulfur, □ Diet 2: low sulfur. Data are presented as the mean of triplicate PCR assays and error bars represent the standard deviation of the mean. Significance values for comparisons of Diet 1 vs Diet 2: * $P < 0.05$.

Overall, the Group 1 and 2 SRB were also significantly lower in numbers than the Group 3 SRB; more than three logs difference in total numbers of cells. Estimation of total SRB in swine manure was 5.53×10^6 for Diet 1 and 5.32×10^6 for Diet 2 (Fig. 4).

Discussion

The use of real-time PCR to quantify microbial populations in complex environmental samples offers an alternative and more sensitive approach to previous, cultivation-based methods. Cultivation, isolation and enumeration of anaerobes can be time consuming and often provides an inaccurate representation of the microbial population. These classic culture-dependent techniques can be strongly biased, often selecting for species that are more amenable to cultivation, and therefore identification. It has been estimated that only a small fraction (0.01–10%) of the micro-organisms present in an environmental sample can be identified using culture-based techniques (Ward *et al.* 1990; Amann *et al.* 1995). Real-time PCR is an extremely sensitive molecular technique for detecting target DNA. The two main approaches include the use of nonspecific binding dyes such as SYBR Green, or the use of highly specific hybridization probes, that bind to complementary target DNA. In the first approach, the primers and amplification conditions have to be highly specific to avoid synthesis of nonspecific target DNA. The second approach uses a set of primers for amplification, and a highly specific probe that will emit a signal upon interaction with the target DNA, enhancing the specificity of the reaction.

In the current study, we used a SYBR Green-based real-time PCR approach to target the *dsrA* gene of SRB in swine manure. It is estimated that SRB can represent up to 7% of the total population of swine manure (Manz *et al.* 1998; Cook *et al.* 2008), therefore representing just

a small proportion of the total population. Previous studies using direct 16S rDNA gene sequencing demonstrated that the predominant bacterial populations of the swine gastrointestinal tract and stored swine manure are low G+C, Gram-positive anaerobic bacteria such as *Clostridium* sp., *Streptococcus* sp. and *Lactobacillus* sp (Whitehead and Cotta 2001; Leser *et al.* 2002). The approach used in the current study facilitates the detection and enumeration of SRB in swine manure that have previously escaped detection using classical culture-based techniques. While SRB are not numerically dominant in manure, they are particularly significant because they produce H_2S , a malodorous and potentially toxic gas, as an end-product of their metabolism.

In the current study, degenerate primer sets were designed to target 100% of the Group 1, 2 and 3 *dsrA* clones isolated by Cook *et al.* from swine manure. In a comparative reaction using the new Group 1 degenerate primer sets with either the previously used TaqMan[®] *dsrA* probe, or with just SYBR Green in the reaction mix, the SYBR Green assay was as sensitive and specific for Group 1 SRB enumeration from swine manure as with using the probe approach. Given these results, and the absence of nonspecific target DNA amplification as evidenced from melt curve analysis, we are confident that the newly designed degenerate primers in conjunction with a SYBR Green-based real-time PCR assay, provide us with a sensitive and efficient method for detecting and enumerating SRB from swine manure. The probe used in the previous study was only complementary to 54% of the SRB clones; therefore by switching to a SYBR Green-based approach, we have circumvented the problem of probe mismatches among clones within and between groups. This approach has also increased the target population of SRB, enabling a more accurate quantification of the SRB in swine manure.

The primer set DSR1F+ and DSR-R has been used to target the *dsrA* gene and enumerate the SRB population in estuarine and marine sediments (Kondo *et al.* 2004; Leloup *et al.* 2007). This primer set was designed to target consensus sequences within an alignment of 76 *dsr* sequences from reference SRB available from GenBank/EMBL/DDBJ nucleotide databases, and resulted in amplification of a 221 bp PCR product (Kondo *et al.* 2004). The authors of the Leloup study determined, using Probe match (ARB), that 112 out of 356 known *dsrA* sequences from SRB have perfectly matched target sites for this primer set. Most of these sequences are from Desulfovibrionaceae and Desulfobacteraceae, whereas most Gram-positive SRB and *Archaeoglobus* species do not have complementary regions to these primers (Leloup *et al.* 2007). Currently, for the purpose of real-time PCR, this primer set provides the most comprehensive coverage

of target *dsrA* genes from known sequences of SRB, but has, to date, only been applied to studies of estuarine and marine environments. In the current study, this primer set was used to amplify the *dsrA* gene of SRB from swine manure. A clone library was constructed and phylogenetic analysis of the sequenced 221 bp PCR product determined that most of the sequenced clones fall within the *Desulfovibrio* lineage. Many of the clones are quite distant from known sequences, suggesting they represent novel species, yet to be isolated and described. In a study by Whitehead and Cotta (2001), approximately half of the sequenced 16S rDNA clones from swine faeces and slurry were <95% similar to any known species in Genbank, suggesting the existence of a large number of unidentified organisms in these environments (Whitehead and Cotta 2001). Similarly, Snell-Castro *et al.* (2005) reported that only 36% of 202 cloned bacterial 16S rDNA sequences from swine manure were >97% similar to known database sequences. One hundred per cent of the Group 1, 2 and 3 *dsrA* clones isolated and sequenced in the Cook study were <85% similar to known Genbank database sequences (Cook *et al.* 2008). The data from the current study, together with data from the Cook study, suggest that the SRB population in swine manure has extensive diversity, much of which is undefined. Further research is needed to identify and isolate these micro-organisms in order to delineate their ecological role in this complex microbial community and to correlate these populations with the production of odorous compounds and other emissions. A few of the sequences in the clone library fell within the *Desulfobulbus* lineage. The predominance of clones within the *Desulfovibrio* and *Desulfobulbus* lineage correlates well with previous studies describing SRB populations in swine manure. Indeed, Cook *et al.* (2008) described 93% of cloned *dsrA* genes from swine manure having 80–85% similarity to either *Desulfovibrio* sp. or *Desulfobulbus* sp. *Desulfovibrio* sp. and *Desulfobulbus* sp. are also both frequently found in waste water treatment systems, wastewater biofilms and activated sludge, suggesting a correlation with the predominant SRB population of manure and other waste systems (Nanninga and Gottschal 1987; Manz *et al.* 1998; Ito *et al.* 2002).

The DSR1F+ and DSR-R primer set not only amplified a diverse and previously unidentified population of SRB from swine manure, but also amplified a higher target SRB population than our Group primers (Fig. 3). The total SRB amplified from Farm 1 swine manure with this primer set was 1.6×10^8 cells ml⁻¹, which was one and a half logs higher than the cumulative number derived from Group amplification (7.3×10^6 cells ml⁻¹). Our data estimated a mean total bacterial count of 4.64×10^{10} cells ml⁻¹ manure, thereby suggesting that the SRB population in this manure sample comprised c. 0.34% of

the total bacterial population. In the Farm 1 manure sample, the individual Group primer sets detected between 1.28×10^6 and 3.71×10^6 cells ml⁻¹, suggesting the populations of these groups of SRB were fairly equal within the manure from this location. Although the DSR1F+ and DSR-R primer set of Kondo target and amplify a larger population of SRB than the Group primers, they cannot discern between changes in different populations of SRB. The Kondo primer set also has limited ability to amplify *Desulfobulbus*-like SRB, which comprise a significant proportion of the population in swine manure. The complexity of changes and differences in SRB populations is exemplified in Fig. 4. In contrast to the fairly equal populations of the SRB Groups in Farm 1 manure (Fig. 3), Group 3 is by far the dominant SRB Group in the manure obtained from the diet studies (Fig. 4). A total of 5.83×10^6 and 3.46×10^6 cells ml⁻¹ of Group 3 SRB for Diets 1 and 2, respectively. Conversely, Groups 1 and 2 were present in much lower numbers, falling within the lower level detection limits of our assays. The population of Group 2 SRB was estimated to be a little $<1 \times 10^3$ cells ml⁻¹ manure for both diets.

For the Group 1 SRB, there was 2.62×10^3 cells ml⁻¹ manure in the control diet. In the low sulfur diet, the population of SRB was estimated at 2.79×10^2 cells ml⁻¹ manure, suggesting that a change in the dietary sulfur content may affect the levels of this Group of SRB in the manure. Group 1 SRB are most similar to the *Desulfobulbus* genera, whereas Group 2 and 3 have similarity to the *Desulfovibrio* sp. Both *Desulfobulbus* sp. and *Desulfovibrio* sp. are able to utilize a wide variety of substrates as electron donors for sulfate reduction (Widdel 1988). In swine manure, the dominant SRB group may be determined by substrate availability. It is tempting to speculate that the lower levels of sulfur in Diet 2 preclude growth of the *Desulfobulbus*-like Group 1, and that the *Desulfovibrio*-like Group 3 can out-compete other SRB for substrates, electron donors and nutrients in the early stages of ecosystem development in the manure. The lower sulfur content of Diet 2 had no effect on the Group 2 and 3 *Desulfovibrio*-like SRB populations. The results presented here are from preliminary studies, but do warrant further investigation into the effects of sulfate availability on SRB populations. In the previous study by Cook *et al.*, it was hypothesized that the dominant SRB population would vary with both the time that the manure is in the storage system and by the nutrient make up of the feed. Data presented in the current study support this hypothesis. The manure samples collected from the dietary experiments had only reached 28 days of maturity, compared with the Farm 1 manure pit sample, which has had months to mature and establish populations of micro-organisms. Data in this study suggest that Group 3 SRB

dominate early in the manure ecosystem, whereas Groups 1 and 2 are present in much lower numbers in the early stages and may take time to establish themselves in manure (Fig. 4). In contrast, the results presented in Fig. 3 suggest that all Groups are represented in fairly equal numbers and comprise a more uniform population of SRB in manure from established pits. This may be attributed to differences in the physiological and ecological preferences of the different genera and species within the SRB population, and the ability to compete for electron donors, substrates and nutrients.

These data demonstrate the usefulness of the Group primer sets in quantifying the SRB populations of swine manure. While the Kondo primers amplify a larger SRB population than our Group primers, and are useful for estimating the total SRB population in the environmental sample, this primer set is unable to distinguish between shifts of SRB within a community. The complexity of SRB populations is also illustrated in this study. In an established manure ecosystem (Farm 1), there is an apparent population of SRB that escapes detection by our Group primer sets, but is amplified by the Kondo primer set. This is suggested by the difference in comparison of numbers of SRB quantified by using the Kondo primer set or the cumulative enumeration of the Groups (one and a half logs difference). In contrast, the total number of SRB amplified from the Diet 1 manure sample was 5.53×10^6 cells ml⁻¹, which is effectively the same number of SRB quantified by the sum of the Group primer sets (5.83×10^6 cells ml⁻¹ SRB manure from Diet 1). This suggests that the diversity of the population of SRB in the manure from the diet studies is equally covered by amplification with either the Kondo primer set or by our Group primers, although the limited amplification of *Desulfobulbus*-like SRB with the Kondo primer set needs to be considered. In the more established ecosystem manure sample from Farm 1, the SRB population may be more diverse and in higher numbers, a proportion of which is only detected by the Kondo primer set.

The aims of this study were to further develop a rapid, simple and sensitive method to enumerate and discern different Groups of SRB in swine manure. The diversity and coverage of these Group primer sets were compared with that of a primer set that had previously only been used to detect SRB from marine environments. This study supports the idea that multiple primer sets are needed to fully investigate the SRB population within a given environmental sample. Further studies are needed to fully investigate the undefined diversity of SRB in swine manure and to correlate the presence of these target groups of SRB with the production of H₂S. Hopefully, this will lead to a more comprehensive understanding of the ecology of SRB populations in manure systems and ultimately lead

to new techniques to control and reduce the production of malodorous emissions.

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References

- Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* **59**, 143.
- Clark, O.G., Morin, B., Zhang, Y., Sauer, W.C. and Feddes, J.J. (2005a) Preliminary investigation of air bubbling and dietary sulfur reduction to mitigate hydrogen sulfide and odor from swine waste. *J Environ Qual* **34**, 2018–2023.
- Clark, O.G., Moehn, S., Edeogu, I. and Leonard, J. (2005b) Manipulation of dietary protein and nonstarch polysaccharide to control swine manure emissions. *J Environ Qual* **34**, 1461–1466.
- Cook, K.L., Whitehead, T.R., Spence, C. and Cotta, M.A. (2008) Evaluation of the sulfate-reducing bacterial population associated with stored swine slurry. *Anaerobe* **14**, 172–180.
- Cotta, M.A., Whitehead, T.R. and Zeltwanger, R.L. (2003) Isolation, characterization and comparison of bacteria from swine faeces and manure storage pits. *Environ Microbiol* **5**, 737–745.
- Dhillon, A., Teske, A., Dillon, J., Stahl, D.A. and Sogin, M.L. (2003) Molecular characterization of sulfate-reducing bacteria in the Guaymas Basin. *Appl Environ Microbiol* **69**, 2765–2772.
- Donham, K.J. (2000) The concentration of swine production. Effects on swine health, productivity, human health, and the environment. *Vet Clin North Am Food Anim Pract* **16**, 559–597.
- Foti, M., Sorokin, D.Y., Lomans, B., Mussman, M., Zacharova, E.E., Pimenov, N.V., Kuenen, J.G. and Muyzer, G. (2007) Diversity, activity, and abundance of sulfate-reducing bacteria in saline and hypersaline soda lakes. *Appl Environ Microbiol* **73**, 2093–2100.
- Harms, G., Layton, A.C., Dionisi, H.M., Gregory, I.R., Garrett, V.M., Hawkins, S.A., Robinson, K.G. and Sayler, G.S. (2003) Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. *Environ Sci Technol* **37**, 343–351.
- Hill, J.E., Hemmingsen, S.M., Goldade, B.G., Dumonceaux, T.J., Klassen, J., Zijlstra, R.T., Goh, S.H. and Van Kessel, A.G. (2005) Comparison of ileum microflora of pigs fed corn-, wheat-, or barley-based diets by chaperonin-60 sequencing and quantitative PCR. *Appl Environ Microbiol* **71**, 867–875.

- Ito, T., Okabe, S., Satoh, H. and Watanabe, Y. (2002) Successional development of sulfate-reducing bacterial populations and their activities in a wastewater biofilm growing under microaerophilic conditions. *Appl Environ Microbiol* **68**, 1392–1402.
- Kjeldsen, K.U., Loy, A., Jakobsen, T.F., Thomsen, T.R., Wagner, M. and Ingvorsen, K. (2007) Diversity of sulfate-reducing bacteria from an extreme hypersaline sediment, Great Salt Lake (Utah). *FEMS Microbiol Ecol* **60**, 287–298.
- Klappenbach, J.A., Saxman, P.R., Cole, J.R. and Schmidt, T.M. (2001) rrndb: the ribosomal RNA operon copy number database. *Nucleic Acids Res* **29**, 181–184.
- Klein, M., Friedrich, M., Roger, A.J., Hugenholtz, P., Fishbain, S., Abicht, H., Blackall, L.L., Stahl, D.A. *et al.* (2001) Multiple lateral transfers of dissimilatory sulfite reductase genes between major lineages of sulfate-reducing prokaryotes. *J Bacteriol* **183**, 6028–6035.
- Kondo, J., Nedwell, D.B., Purdy, K.J. and de Queiroz Silva, S. (2004) Detection and enumeration of sulphate-reducing bacteria in estuarine sediments by competitive PCR. *Geomicrobiol J* **21**, 145–157.
- Leloup, J., Quillet, L., Berthe, T. and Petit, F. (2006) Diversity of the dsrAB (dissimilatory sulfite reductase) gene sequences retrieved from two contrasting mudflats of the Seine estuary, France. *FEMS Microbiol Ecol* **55**, 230–238.
- Leloup, J., Loy, A., Knab, N.J., Borowski, C., Wagner, M. and Jorgensen, B.B. (2007) Diversity and abundance of sulfate-reducing microorganisms in the sulfate and methane zones of a marine sediment, Black Sea. *Environ Microbiol* **9**, 131–142.
- Leser, T.D., Amenuvor, J.Z., Jensen, T.K., Lindecrona, R.H., Boye, M. and Moller, K. (2002) Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Appl Environ Microbiol* **68**, 673–690.
- Manz, W., Eisenbrecher, M., Neu, T.R. and Szwedzyk, U. (1998) Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ probing with specific 16S rRNA targeted oligonucleotides. *FEMS Microbiol Ecol* **25**, 43–61.
- Minz, D., Flax, J.L., Green, S.J., Muyzer, G., Cohen, Y., Wagner, M., Rittmann, B.E. and Stahl, D.A. (1999) Diversity of sulfate-reducing bacteria in oxic and anoxic regions of a microbial mat characterized by comparative analysis of dissimilatory sulfite reductase genes. *Appl Environ Microbiol* **65**, 4666–4671.
- Nanninga, H.J. and Gottschal, J.C. (1987) Properties of *Desulfovibrio carbinolicus* sp. nov. and other sulfate-reducing bacteria isolated from an anaerobic-purification plant. *Appl Environ Microbiol* **53**, 802–809.
- Odom, J.M. and Peck, H.D. Jr (1984) Hydrogenase, electron-transfer proteins, and energy coupling in the sulfate-reducing bacteria *Desulfovibrio*. *Annu Rev Microbiol* **38**, 551–592.
- Panetta, D.M., Powers, W.J., Xin, H., Kerr, B.J. and Stalder, K.J. (2006) Nitrogen excretion and ammonia emissions from pigs fed modified diets. *J Environ Qual* **4**, 1297–1308.
- Perez-Jimenez, J.R., Young, L.Y. and Kerkhof, L.J. (2001) Molecular characterization of sulfate-reducing bacteria in anaerobic hydrocarbon-degrading consortia and pure cultures using the dissimilatory sulfite reductase (dsrAB) genes. *FEMS Microbiol Ecol* **35**, 145–150.
- Snell-Castro, R., Godon, J.J., Delgenés, J.-P. and Dabert, P. (2005) Characterisation of the microbial diversity in a pig manure storage pit using small subunit rDNA sequence analysis. *FEMS Microbiol Ecol* **52**, 229–242.
- Sutton, A.L., Kephart, K.B., Verstegen, M.W., Canh, T.T. and Hobbs, P.J. (1999) Potential for reduction of odorous compounds in swine manure through diet modification. *J Anim Sci* **77**, 430–439.
- Tajima, K., Nonaka, I., Higuchi, K., Takusari, N., Kurihara, M., Takenaka, A., Mitsumori, M., Kajikawa, H. *et al.* (2007) Influence of high temperature and humidity on rumen bacterial diversity in Holstein heifers. *Anaerobe* **13**, 57–64.
- Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Wagner, M., Roger, A.J., Flax, J.L., Brusseau, G.A. and Stahl, D.A. (1998) Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. *J Bacteriol* **180**, 2975–2982.
- Ward, D.M., Weller, R. and Bateson, M.M. (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* **345**, 63–65.
- Whitehead, T.R. and Cotta, M.A. (2001) Characterisation and comparison of microbial populations in swine faeces and manure storage pits by 16S rDNA gene sequence analyses. *Anaerobe* **7**, 181–187.
- Whitehead, T.R. and Cotta, M.A. (2004) Isolation and identification of hyper-ammonia producing bacteria from swine manure storage pits. *Curr Microbiol* **48**, 20–26.
- Whitehead, T.R., Cotta, M.A., Collins, M.D. and Lawson, P.A. (2004) *Hespellia stercorisuis* gen. nov., sp. nov. and *Hespellia porcina* sp. nov., isolated from swine manure storage pits. *Int J Syst Evol Microbiol* **54**, 241–245.
- Whitehead, T.R., Cotta, M.A., Collins, M.D., Falsen, E. and Lawson, P.A. (2005) *Bacteroides coprosuis* sp. nov., isolated from swine manure storage pits. *Int J Syst Evol Microbiol* **55**, 2515–2518.
- Whitford, M.F., Forster, R.J., Beard, C.E., Gong, J. and Teather, R.M. (1998) Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. *Anaerobe* **4**, 153–163.
- Widdel, F. (1988) Microbiology and ecology of sulfate- and sulfur-reducing bacteria. In *Biology of Anaerobic Microorganisms* ed. Zehnder, A.J.B. pp. 469–585. New York: John Wiley & Sons.
- Zahn, J.A., Hatfield, J.L., Do, Y.S., DiSpirito, A.A., Laird, D.A. and Pfeiffer, R.L. (1997) Characterization of volatile organic emissions and wastes from a swine production facility. *J Environ Qual* **26**, 1687–1696.